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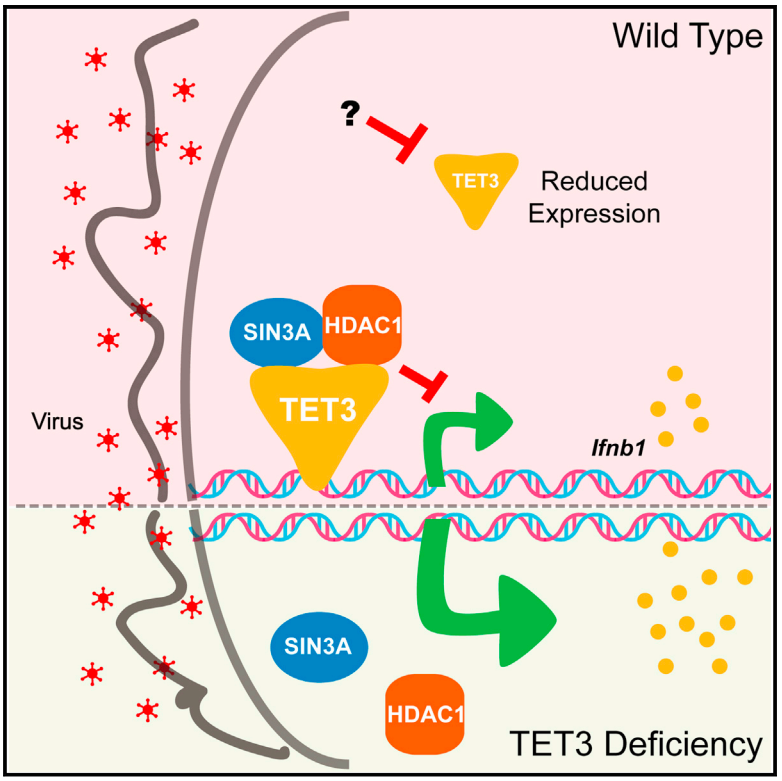
Article

TET3 Inhibits Type I IFN Production Independent of DNA Demethylation

Graphical Abstract



Authors

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In Brief

Xue et al. find that TET3 expression is decreased after viral infection and that TET3 reduction enhances type I IFN production and virus clearance. Mechanistically, TET3 recruits HDAC1 to the *Ifnb1* promoter, thus suppressing type I IFN transcription.

Highlights

- TET3 expression is decreased upon virus stimulation
- TET3 decreases type I IFN production after poly(I:C) stimulation or viral infection
- TET3 suppresses type I IFN production independent of DNA demethylation
- TET3 recruits HDAC1 to the promoter of *Ifnb1*

Xue et al., 2016, Cell Reports *16*, 1096–1105
July 26, 2016
http://dx.doi.org/10.1016/j.celrep.2016.06.068

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TET3 Inhibits Type I IFN Production Independent of DNA Demethylation

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http://dx.doi.org/10.1016/j.celrep.2016.06.068

SUMMARY

Type I interferons (IFNs) play both beneficial and harmful roles in antiviral responses. Precise regulation of host type I IFNs is thus needed to prevent immune dysregulation. Here, we find that the DNA demethylase TET3 is a negative regulator of IFN-β in response to poly(I:C) stimulation or viral infection. Deletion of TET3 enhances antiviral responses, with elevated expression of IFN-β and IFN-stimulated genes. The catalytic domain of TET3 was critical for the suppression of IFN-β production, but TET3 enzymatic activity was dispensable. Instead, the catalytic domain of TET3 interacts with HDAC1 and SIN3A, thus enhancing their binding to the *Ifnb1* promoter. Our study demonstrates that TET3 negatively regulates type I IFN production independent of DNA demethylation. This not only sheds light on TET3 as a signaling protein in immune cells for gene regulation but also will help to develop strategies to prevent type I IFN-related disease.

INTRODUCTION

Type I interferons (IFNs) include IFN-α and IFN-β. Accumulating evidence suggests that type I IFNs play multifaceted roles in diseases (Trinchieri, 2010). Initially, type I IFNs were discovered as critical antiviral cytokines, and recent reports suggest that the blockade of type I IFN signaling in virus-induced chronic infection could decrease immune suppression and accelerate the clearance of persistent infections (Teijaro et al., 2013; Wilson et al., 2013). During bacterial infections, type I IFNs could protect the host through the production of proinflammatory cytokines, but type I IFNs are also suggested to be hijacked by *Listeria* to induce exaggerated apoptosis (Carrero et al., 2004). In addition to infectious diseases, type I IFNs might also act as double-edged swords in autoimmune diseases. Excessive interferon is recognized as a prominent aspect in patients with systemic lupus erythematosus (SLE) (Rönnblom et al., 2009), and IFN-β is used as a drug to treat multiple sclerosis (Comi, 2009; Noseworthy et al., 2000). Therefore, precise regulation of type I IFN production is critical in order to maintain host homeostasis.

The production of type I IFN could be modulated in various ways. Post-translational modifications, including phosphorylation and ubiquitination, are critical for modulating signal transduction during type I IFN production (Richards and Macdonald, 2011). Notably, epigenetic modifiers are among the key players at the transcriptional level for type I IFN regulation, altering chromatin accessibility for transcription factors (Ford and Thanos, 2010). Histone modifications such as acetylation and methylation could be efficiently switched on and off, which makes histones ideal for efficiently regulating gene transcription (Álvarez-Errico et al., 2015). As for DNA modifications, methylated cytosine in the promoter region has been suggested for transcriptional silencing (Schübeler, 2015), and TET family members have been recently discovered as new DNA demethylases for gene activation (Hu et al., 2015; Tahiliani et al., 2009).

The TET family of proteins, including TET1, TET2 and TET3, can catalyze the oxidation of the methyl group on cytosine (He et al., 2011). TET family members have been shown to participate in numerous development processes (Branco et al., 2012), including hematopoiesis. TET2 mutations are contributors to hematologic neoplasms, and mouse studies have suggested that mutated TET2 results in aberrant common myeloid progenitor (CMP) differentiation (Figueroa et al., 2010; Moran-Crusio et al., 2011; Quivoron et al., 2011). In addition, TET1 has been suggested as a tumor suppressor in non-Hodgkin B cell lymphoma (Cimmino et al., 2015). As noted previously, emerging evidence has demonstrated that the TET family also has an essential role in immune cells. In T cells, TET2 is able to regulate FoxP3 expression and affects the differentiation and homeostasis of $CD4^+FoxP3^+$ regulatory T cells (Yang et al., 2015). In myeloid cells, a recent discovery shows that TET2 is involved in interleukin 6 (IL-6) production (Zhang et al., 2015). TET3 is also expressed in immune cells, but its role in the production of type I IFN by immune cells is undefined.

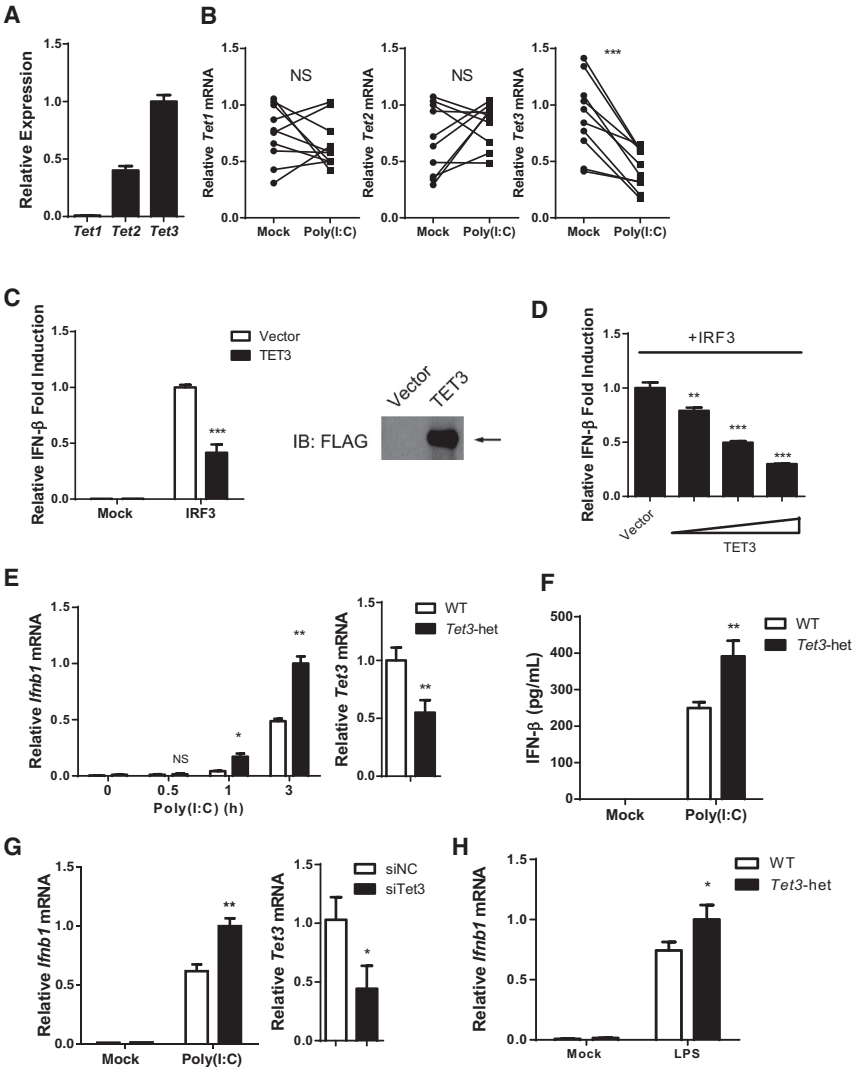

Figure 1. TET3 Suppresses IFN-β Production via the TLR3 Pathway

(A) Relative mRNA expression of *Tet1*, *Tet2*, and *Tet3* in BMDMs.

(B) Paired analysis of the relative mRNA expression of *Tet1* (left), *Tet2* (middle), and *Tet3* (right) in PEMs that were unstimulated (Mock) or stimulated with poly(I:C).

(C) Relative IFN-β luciferase activity in HEK293T cells transfected with TET3 or the empty control vector, with or without the constitutively active form of IRF3. Arrow indicates TET3 protein levels (right).

(D) Relative IFN-β luciferase activity in HEK293T cells transfected with vector plasmid (1,000 ng) or various concentrations of TET3 plasmids (10 ng, 100 ng, or 1,000 ng), along with the constitutively active form of IRF3.

(E) The relative mRNA expression of *Ifnb1* in WT and *Tet3*-het BMDMs stimulated with poly(I:C) for the indicated time. Relative mRNA expression of *Tet3* in WT and *Tet3*-het BMDMs (right).

(F) IFN-β concentrations in the supernatants of WT and *Tet3*-het BMDMs that were unstimulated or stimulated with poly(I:C) for 16 hr.

(G) The relative mRNA expression of *Ifnb1* in the control or Tet3-knockdown PEMs stimulated with poly(I:C). Right panel shows the knockdown efficiency.

(H) Relative mRNA expression of *Ifnb1* in WT and *Tet3*-het BMDMs after LPS stimulation.

Error bars represent the mean ± SD of at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

Here, we provide evidence that TET3 inhibited the production of type I IFNs, especially IFN-β, and deletion of TET3 in macrophages enhanced antiviral responses. Suppression of IFN-β production by TET3 was mediated by the HDAC1-SIN3A module, which was independent of the DNA demethylation activity of TET3.

RESULTS

TET3 Suppresses IFN-β Production via the TLR3 Pathway

To investigate the role of TET family members in IFN-β production, we first analyzed the mRNA expression levels of *Tet1, Tet2*, and *Tet3* in primary murine bone-marrow-derived macrophages (BMDMs). TET3 showed the highest expression level among TET family members, while TET1 was barely expressed in macrophages (Figure 1A). The Toll-like receptor 3 (TLR3) pathway is a well-characterized pathway producing type I IFNs and is critical in host defense against various viruses (Hoebe et al., 2003; Li et al., 2005; Zhang et al., 2007). We stimulated murine BMDMs with poly(I:C) to activate the TLR3 pathway. mRNA levels of *Tet3*, rather than *Tet1* and *Tet2*, were significantly decreased compared to those of the unstimulated BMDMs (Figure 1B). We therefore asked whether TET3 might regulate TLR3-mediated type I IFN production. When compared to poly(I:C)-treated wild-type (WT) macrophages, mRNA levels of *Ifnb1*, which encodes IFN-β, were not significantly changed in *Tet1* knockout (KO) macrophages (Figure S1A). Because we did not have *Tet2* KO mice and *Tet3* KO mice are embryonic lethal, we transfected HEK293T cells with plasmids expressing TET2 or TET3 together with the IFN-β luciferase reporter to evaluate IFN-β levels. The constitutively active form of IRF3 was also transfected into these HEK293T cells to stimulate IFN-β production, as previously described (Li et al., 2015b). Compared to the vector control cells, overexpression of TET2 showed comparable IFN-β induction (Figure S1B); in contrast, overexpression of TET3 significantly reduced IFN-β luciferase readings (Figure 1C). When TET3 overexpression levels were gradually increased, inhibition of IFN-β induction was dose dependent (Figure 1D). Because TET3 deficiency results in embryonic lethality in mice, we next used primary BMDMs from WT and *Tet3*-heterozygous (*Tet3*-het) mice (Figure 1E; the mRNA levels of *Tet3* are shown in the right panel).

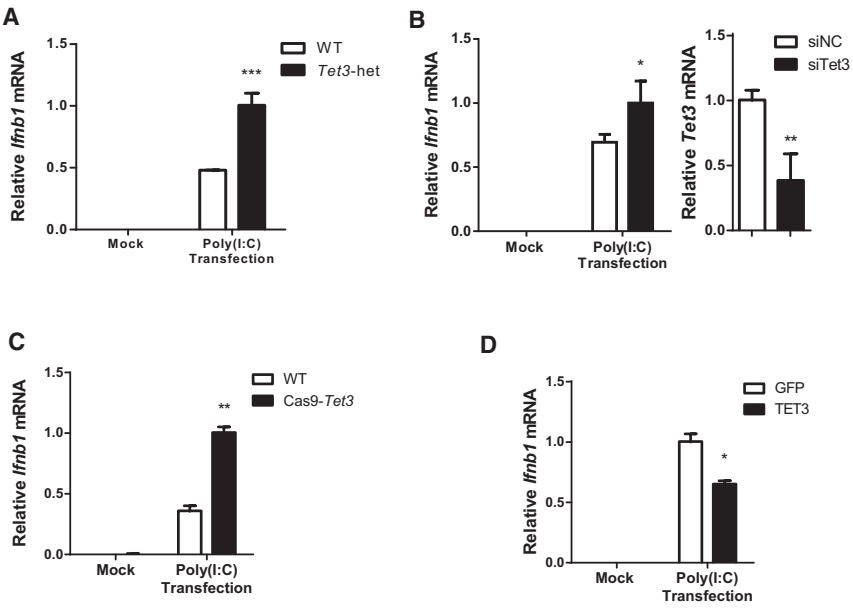

Figure 2. TET3 Suppresses IFN-β Production via the RIG-I/MDA-5 Pathway

(A) Relative mRNA expression of *Ifnb1* in WT and *Tet3*-het BMDMs after poly(I:C) transfection.

(B) Relative mRNA expression of *Ifnb1* in control or Tet3-knockdown PEMs transfected with poly(I:C). Right panel indicates the knockdown efficiency.

(C) MEFs targeted with control sgRNA (WT) or *Tet3* Cas9-sgRNA (Cas9-*Tet3*) were transfected with poly(I:C), and the relative mRNA expression of *Ifnb1* was analyzed.

(D) Relative mRNA expression of *Ifnb1* in primary MEFs overexpressing GFP or TET3 after transfection with poly(I:C).

Error bars represent the mean ± SD of at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

Tet3-het mice generated percentages of splenic macrophages, peritoneal macrophages (PEMs), or BMDMs similar to WT mice, as indicated by $CD11b^+F4/80^+$ staining (Figure S2A). The populations of $CD4^+$ T cells, $CD8^+$ T cells and $B220^+$ B cells were also normal in the spleens of *Tet3*-het mice (Figure S2B). In response to poly(I:C) stimulation, IFN-β transcripts were indeed elevated in *Tet3*-het macrophages compared with WT BMDMs, which was observed as early as 1 hr after treatment (Figure 1E, left). ELISAs showed that IFN-β concentrations were also increased in the supernatants of poly(I:C)-treated *Tet3*-het macrophages (Figure 1F). In addition, knockdown (KD) of Tet3 in PEMs using specific small interfering RNA (siRNA) enhanced IFN-β production upon poly(I:C) stimulation (Figure 1G; Tet3 KD efficiency is shown in the right panel). In contrast, minor differences in IFN-β production were observed between lipopolysaccharide (LPS)-stimulated *Tet3*-het and WT macrophages (i.e., TLR4 pathway) (Figure 1H). Together, these data suggest that reduced TET3 expression enhances IFN-β production in macrophages upon activation of the TLR3 pathway.

TET3 Inhibits IFN-β Production via the RIG-I/MDA-5 Pathway

In addition to TLR3/TLR4, retinoic acid-inducible gene I and melanoma differentiation-associated gene 5 (RIG-I/MDA-5), receptors localized in the cytosol, can also induce type I IFNs against infection in macrophages (Trinchieri, 2010). We next examined whether TET3 regulated IFN-β production via the RIG-I/MDA5 pathway. As reported previously, transfection of poly(I:C) using Lipofectamine can activate the RIG-I/MDA5 pathway (Li et al., 2015b). We found that the poly(I:C)-transfected *Tet3*-het macrophages had substantially enhanced IFN-β mRNA levels compared to WT cells (Figure 2A). KD of Tet3 in PEMs also increased IFN-β induction upon poly(I:C) transfection (Figure 2B). Next, we used Cas9 single guide RNAs (sgRNAs) to target the *Tet3* gene in mouse embryonic fibroblasts (MEFs) and confirmed the frameshift mutations in both alleles of *Tet3* after sequencing the Cas9-sgRNA-treated MEF cell clone (Figure S3). The poly(I:C)-transfected Cas9-*Tet3* MEFs also enhanced IFN-β production via the RIG-I/MDA5 pathway (Figure 2C). Consistent with these findings, primary MEFs stably overexpressing TET3 revealed a significant suppression of IFN-β transcription after poly(I:C) transfection (Figure 2D). We therefore suggest that TET3 negatively regulates IFN-β production via the TLR3 and RIG-I/MDA5 pathways.

The Enzymatic Activity of TET3 Is Dispensable for the Suppression of IFN-β Transcription

Considering that TET3 is well known for its conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (He et al., 2011; Ito et al., 2010), which is the initial step of active DNA demethylation and generally results in gene activation, we were surprised to observe that TET3 decreased IFN-β production. According to a previous report, TET enhances the expression levels of the *miR-200* family, which then results in repression of *miR-200*-family-targeted genes (Hu et al., 2014). Because miRNA-146a (*miR-146a*) was suggested as a negative regulator of RIG-I-dependent type I IFN production and antiviral responses (Hou et al., 2009; Wu et al., 2013), we assessed whether TET3 enhanced *miR-146a* expression. However, no difference in *miR-146a* expression was observed in WT and *Tet3*-het macrophages (Figure 3A). We next questioned whether the enzymatic activity of TET3, a methylcytosine dioxygenase, was critical for IFN-β production. TET3 contains a catalytic domain in its C terminus and a CXXC domain in its N terminus (Figure 3B, left) (Tan and Shi, 2012). A recent study generated the dioxygenase-dead TET3 mutation (H950Y and D952A, named demethylation mutant) caused by two point mutations in the conserved Fe^{2+}-binding motif (Gu et al., 2011). In addition, we truncated the N terminus of TET3 to generate the catalytic domain (CD) or the catalytic domain containing the H950Y and D952A mutations (Mut-CD). Alternatively, we truncated the catalytic domain in the C terminus of TET3 to generate its N-terminal region

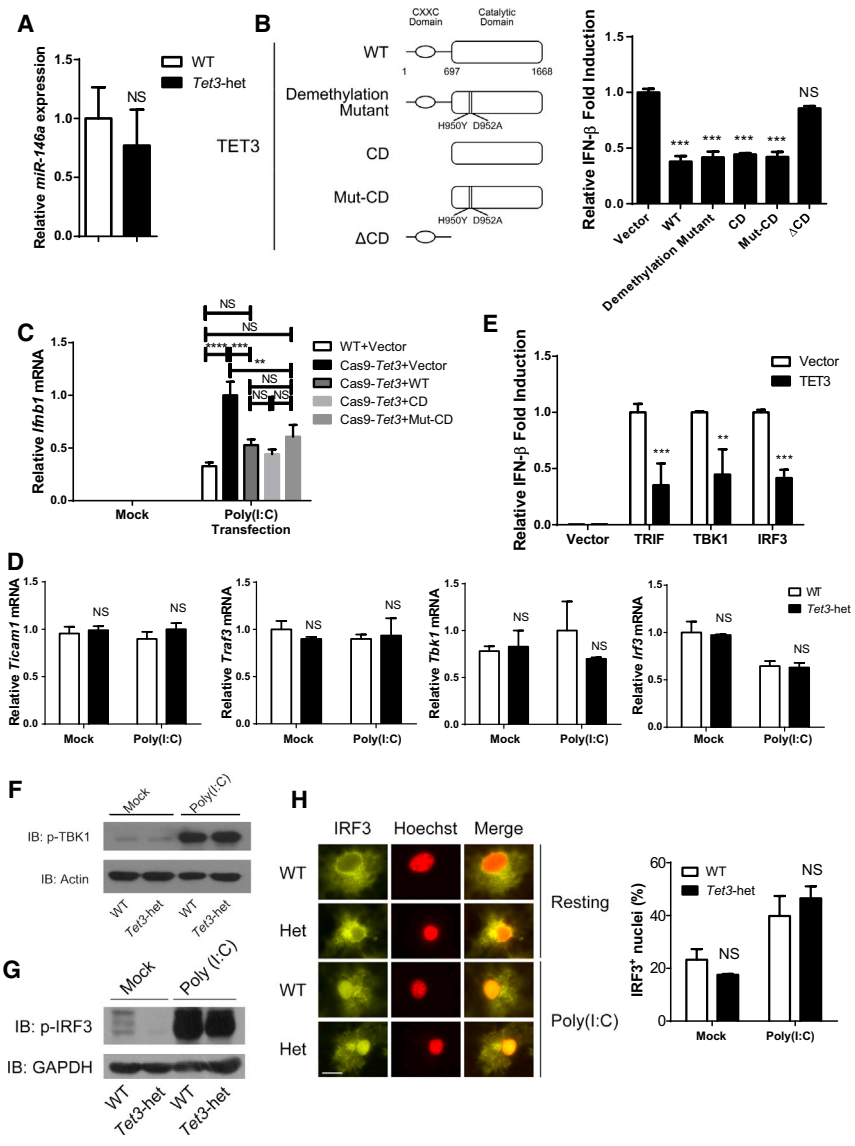

Figure 3. The Enzymatic Activity of TET3 Is Dispensable for the Suppression of IFN-β Transcription

(A) Relative expression of *miR-146a* in WT and *Tet3*-het BMDMs.

(B) Diagram of WT or the mutated or truncated TET3 (left). Relative IFN-β luciferase activity in HEK293T cells transfected with the indicated forms of TET3, along with the constitutively active form of IRF3 (right).

(C) Relative mRNA expression of *Ifnb1* in WT MEFs, Cas9-*Tet3* MEFs, or Cas9-*Tet3* MEFs reconstituted with the indicated forms of TET3 and transfected with poly(I:C).

(D) Relative mRNA levels of *Ticam1* (encodes TRIF), *Traf3*, *Tbk1*, and *Irf3* in WT and *Tet3*-het BMDMs left unstimulated or stimulated with poly(I:C).

(E) Relative IFN-β luciferase activity in HEK293T cells transfected with vector or TET3 together with TRIF, TBK1, or the constitutively active form of IRF3 to activate IFN-β production.

(F) Immunoblotting analysis of p-TBK1 in WT and *Tet3*-het BMDMs left unstimulated or stimulated with poly(I:C).

(G) Immunoblotting analysis of p-IRF3 in WT and *Tet3*-het BMDMs left unstimulated or stimulated with poly(I:C).

(H) Immunofluorescence analysis of IRF3 in WT and *Tet3*-het BMDMs left unstimulated or stimulated with poly(I:C) (left). Percentages of cells showing IRF3$^+$ staining in the nuclei (right). Scale bars represent 10 μm.

Error bars represent the mean ± SD of at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

(named ΔCD). WT TET3 or the TET3 mutants were overexpressed in HEK293T cells to analyze their suppressive function using an IFN-β luciferase assay. The demethylation mutant, CD, and Mut-CD inhibited IFN-β luciferase activity in a manner similar to that of full-length WT TET3 (Figure 3B, right). Moreover, Mut-CD, as well as CD and WT TET3, could reverse enhanced IFN-β production in TET3-deficient MEFs to a level comparable to that found in the WT control MEFs (Figure 3C). In contrast, the ΔCD mutation, which has lost the catalytic domain, failed to inhibit IFN-β induction (Figure 3B, right). This indicates that the catalytic domain of TET3 is critical for IFN-β suppression but the enzymatic activity of TET3 is dispensable.

We next asked which key signaling proteins in the TLR3 or RIG-I/MDA5 pathways were transcriptionally affected by TET3. TET3 deficiency in macrophages did not affect the mRNA levels of *Ticam1* (which encodes TRIF), *Traf3, Tbk1, Irf3*, and *Rela* (which encodes the nuclear factor κB [NF-κB] p65 subunit) when compared to those in WT cells (Figures 3D and S4A). Using the IFN-β luciferase reporter, we found that TET3 suppressed TRIF-, TBK1-, and IRF3-induced IFN-β transcription (Figure 3E). Phosphorylation levels of TBK1 and IRF3 (Figures 3F and 3G), together with the degradation of IκB-α and the phosphorylation levels of p65 (Figure S4B), were also not altered in *Tet3*-het macrophages after poly(I:C) treatment. Consistent with the unaffected canonical NF-κB pathway, poly(I:C)-treated *Tet3*-het macrophages produced levels of IL-6 and TNF-α similar to those produced by WT cells (Figures S4C and S4D). In addition, the amount of nuclear-localized IRF3 was not altered in untreated or poly(I:C)-stimulated *Tet3*-het macrophages (Figure 3H). These data suggest that TET3 might function downstream of IRF3, leading to the key question of whether TET3 directly acts on the *Ifnb1* locus.

The Catalytic Domain of TET3 Binds HDAC1 and SIN3A

We analyzed the mass spectrometry data from TET1 immunoprecipitation (Cartron et al., 2013; Williams et al., 2011) and found that the transcriptional corepressor SIN3A and histone deacetylase 1 (HDAC1) were potential binding partners of TET1. Previous studies revealed that HDAC1 forms a transcriptional

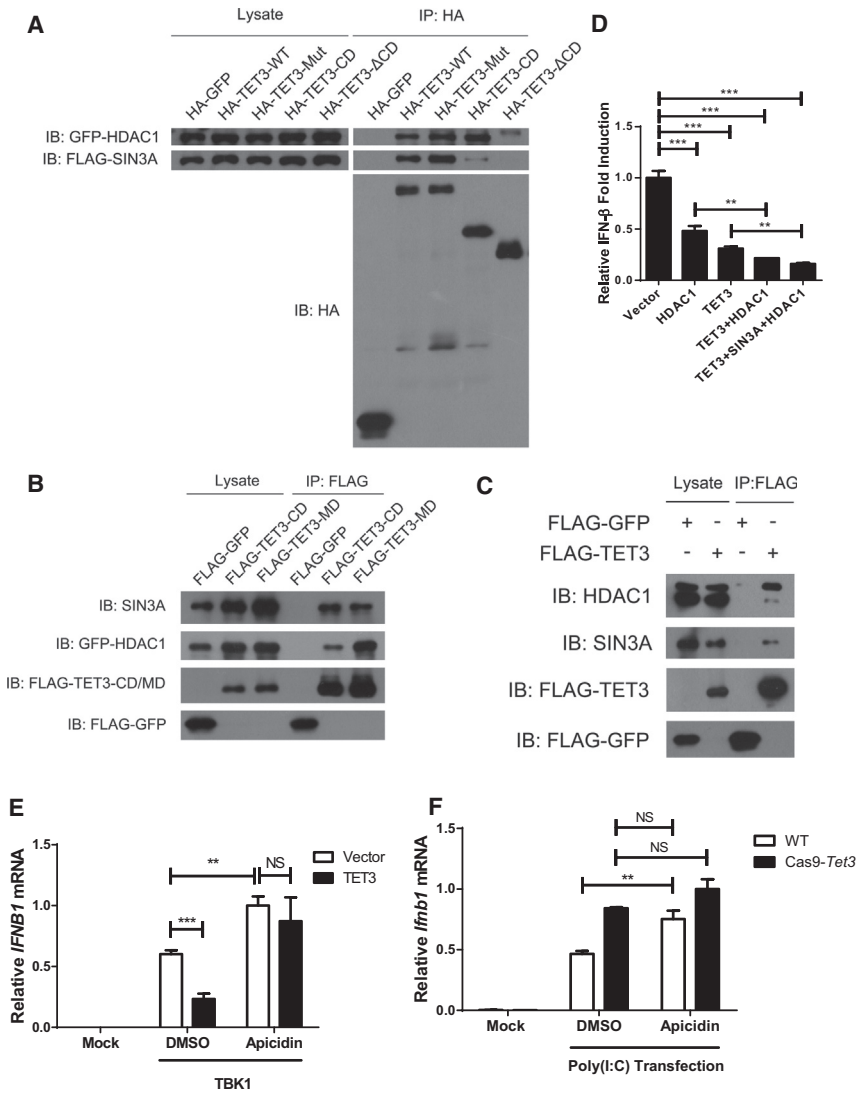

Figure 4. The Catalytic Domain of TET3 Binds HDAC1 and SIN3A

(A) Immunoprecipitation using anti-HA antibody in HEK293T cells transfected with HA-GFP or the HA-tagged indicated form of TET3, together with GFP-HDAC1 and FLAG-SIN3A. Immunoblotting analysis of HA-tagged proteins, GFP-tagged HDAC1, and FLAG-tagged SIN3A was performed.

(B) Immunoprecipitation using anti-FLAG antibody in HEK293T cells transfected with FLAG-GFP or the FLAG-tagged indicated form of TET3, together with GFP-HDAC1. Immunoblotting analysis of FLAG-tagged proteins, GFP-tagged HDAC1, and endogenous SIN3A was performed.

(C) Immunoprecipitation using anti-FLAG antibody in primary MEFs transfected with FLAG-GFP or FLAG-tagged TET3. Immunoblotting analysis of FLAG-tagged proteins and endogenous HDAC1/SIN3A was performed.

(D) The relative IFN-β luciferase activity in HEK293T cells transfected with the indicated plasmids, along with the constitutively active form of IRF3.

(E) HEK293T cells were transfected with the vector plasmid or TET3 plasmid, along with TBK1 plasmid. The cells were treated with DMSO or apicidin (1 μM), and the relative mRNA expression of *IFNB1* was examined.

(F) WT or Cas9-*Tet3*-targeted MEFs were transfected with poly(I:C), together with treatment with DMSO or apicidin (1 μM), and the relative mRNA expression of *Ifnb1* was examined.

Error bars represent the mean ± SD of at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

repressor complex on the *IFNB1* locus (Nusinzon and Horvath, 2006). We therefore examined whether TET3 interacted with SIN3A or HDAC1 to regulate IFN-β transcription. FLAG-tagged TET3 or FLAG-tagged GFP were overexpressed in MEFs that were left unstimulated or transfected with poly(I:C), and the MEFs were then stained with anti-FLAG and anti-HDAC1 (Figure S5). In both unstimulated and poly(I:C)-stimulated MEFs, TET3 was mainly located in the nuclei and colocalized with HDAC1 (Figure S5). Next, we found that the hemagglutinin (HA)-tagged full-length TET3 or its DNA demethylation function-dead mutant (TET3-Mut) could precipitate GFP-fused HDAC1 and FLAG-tagged SIN3A (Figure 4A, HA immunoprecipitation (IP) samples: lane 2 versus lane 3). Furthermore, the catalytic domain of TET3 (TET3-CD), but not its N-terminal domain (TET3-ΔCD), retained the interaction with HDAC1 or SIN3A (Figure 4A, lane 4 versus lane 5). We confirmed that the TET3 Mut-CD mutant could also immunoprecipitate endogenous SIN3A or the transfected GFP-tagged HDAC1 (Figure 4B, FLAG IP samples: lanes 2 and 3). Because the current commercial or self-developed antibodies against TET3 were not able to immunoprecipitate or detect endogenous TET3, we precipitated FLAG-tagged TET3 in primary MEFs and found that TET3 could indeed interact with endogenous HDAC1 and SIN3A (Figure 4C).

Next, we analyzed whether the TET3-HDAC1-SIN3A complex inhibited IFN-β production. Overexpression of HDAC1 reduced IFN-β induction to 48.2%, and co-expression of TET3 further suppressed this to 21.6% (Figure 4D), suggesting a synergistic inhibition effect. Importantly, treatment with apicidin, an HDAC1 inhibitor (Huber et al., 2011), significantly enhanced IFN-β expression in HEK293T cells and also rescued the reduced IFN-β levels in TET3-overexpressing cells (Figure 4E). Next, we examined this phenotype in primary MEFs. Upon poly(I:C) transfection, apicidin treatment augmented IFN-β production in WT MEFs, which reached a level comparable to that found in TET3-deficient MEFs (Figure 4F). These data suggest that TET3 might inhibit IFN-β transcription through HDAC1.

TET3 Promotes the Interaction of HDAC1 or SIN3A with the *Ifnb1* Promoter

Next, we performed chromatin immunoprecipitation (ChIP) assays to address how TET3, HDAC1, and SIN3A cooperated

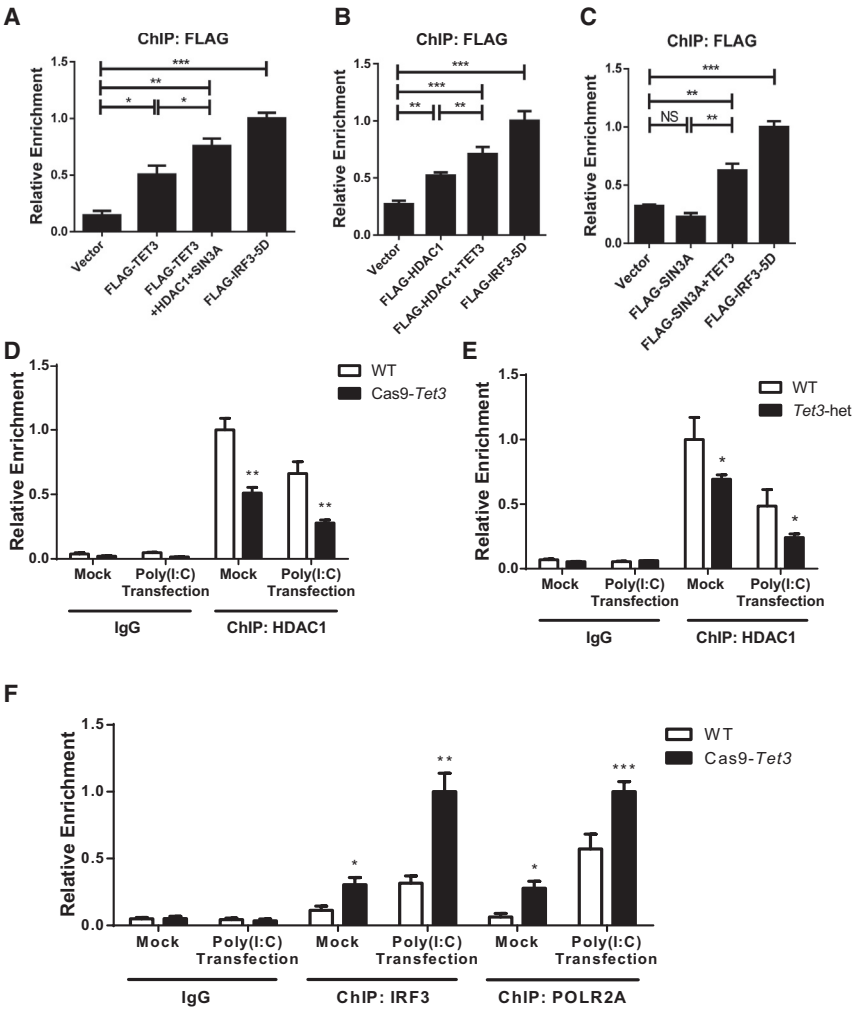

Figure 5. TET3 Promotes the Interaction of HDAC1 or SIN3A with the *Ifnb1* Promoter

(A–C) ChIP assays followed by qPCR for FLAG-tagged (A) TET3, (B) HDAC1, and (C) SIN3A in the *IFNB1* promoter in HEK293T cells transfected with the indicated plasmids. The constitutively active form of IRF3 (FLAG-IRF3-5D) served as a positive control.

(D) ChIP assays followed by qPCR for HDAC1 in the *Ifnb1* promoter in WT or Cas9-*Tet3*-targeted MEFs left unstimulated or transfected with poly(I:C).

(E) ChIP assays followed by qPCR for HDAC1 in the *Ifnb1* promoter in WT or *Tet3*-het PEMs left unstimulated or transfected with poly(I:C).

(F) ChIP assays followed by qPCR for IRF3 and RNA polymerase II in the *Ifnb1* promoter in WT or Cas9-*Tet3*-targeted MEFs left unstimulated or transfected with poly(I:C).

Error bars represent the mean ± SD of at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

and interacted with the *Ifnb1* promoter. The constitutively active form of IRF3 (termed IRF3-5D) was used as a positive control (Figures 5A–5C). To compare the relative amount of TET3, HDAC1, SIN3A, or IRF3-5D enrichment in the *IFNB1* promoter, these molecules were all tagged with FLAG for ChIP assays using anti-FLAG antibodies (Figures 5A–5C). TET3 and HDAC1 were able to bind to the *IFNB1* promoter (Figures 5A and 5B, lane 2). Interestingly, coexpression of HDAC1 and SIN3A further enhanced the binding of TET3 to the *IFNB1* promoter (Figure 5A, lane 3 versus lane 2), and coexpression of TET3 further enhanced the binding of HDAC1 or SIN3A to the *IFNB1* promoter (Figures 5B and 5C, lane 3 versus lane 2). We then tested this phenotype using WT or TET3-deficient cells, which were left untreated or transfected with poly(I:C). The amount of HDAC1 in the *Ifnb1* promoter was decreased in WT MEFs and PEMs upon poly(I:C) transfection, which was in agreement with the inhibitory effect of HDAC1 on IFN-β transcription (Figures 5D and 5E, white bars). Importantly, TET3-deficient MEFs or *Tet3*-het PEMs further removed a significant amount of HDAC1 from the *Ifnb1* promoter (Figures 5D and 5E, black bars), which was correlated with elevated binding of IRF3, RNA polymerase II (POLR2A), and p65 to the *Ifnb1* promoter in TET3-deficient MEFs (Figures 5F and S4E). This suggests that TET3 elevates the occupancy of HDAC1 and SIN3A in the *Ifnb1* promoter for the reduction of histone acetylation, which might result in impaired binding of IRF3 and RNA polymerase II for IFN-β transcription.

TET3 Inhibits Antiviral Responses

To identify the role of TET3 in viral clearance, we first analyzed the expression of TET3 in PEMs after challenge with vesicular stomatitis virus (VSV; an RNA virus). GFP was inserted into the VSV genome, which enabled us to assess the viral load by immunofluorescence microscopy or fluorescence-activated cell sorting (FACS). Consistent with the results in the poly(I:C)-treated macrophages, the mRNA levels of *Tet3* were reduced in the VSV-infected murine macrophages (Figure 6A). Peripheral blood mononuclear cells (PBMCs) from mice infected with influenza virus (A/Puerto Rico/8/1934 H1N1 strain) also had decreased the mRNA levels of *Tet3* (Figure S6A). We then infected WT and *Tet3*-het PEMs with VSV, Sendai virus (SeV; an RNA virus), or herpes simplex virus (HSV; a DNA virus). After infection with these viruses, *Tet3*-het macrophages increased mRNA levels of *Ifnb1* (Figure 6B) as well as *Ifnα2* (Figure 6C, left) compared to WT macrophages. In agreement with this, *Tet3*-het macrophages increased mRNA levels of interferon-stimulated genes (ISGs), including *Mx1*, *Oas1a*, *Ccl5*, and *Cxcl10* (Figure 6C). TET3-deficient MEFs also had increased mRNA levels of *Ifnb1* and *Ifnα2* as well as other antiviral genes upon VSV challenge (Figure S6B). To examine whether TET3 caused primary macrophages to clear viruses, we analyzed the viral load of VSV in infected PEMs. The percentage of VSV-GFP$^+$ was significantly lower in *Tet3*-het PEMs than in WT PEMs as determined by fluorescence imaging analysis or

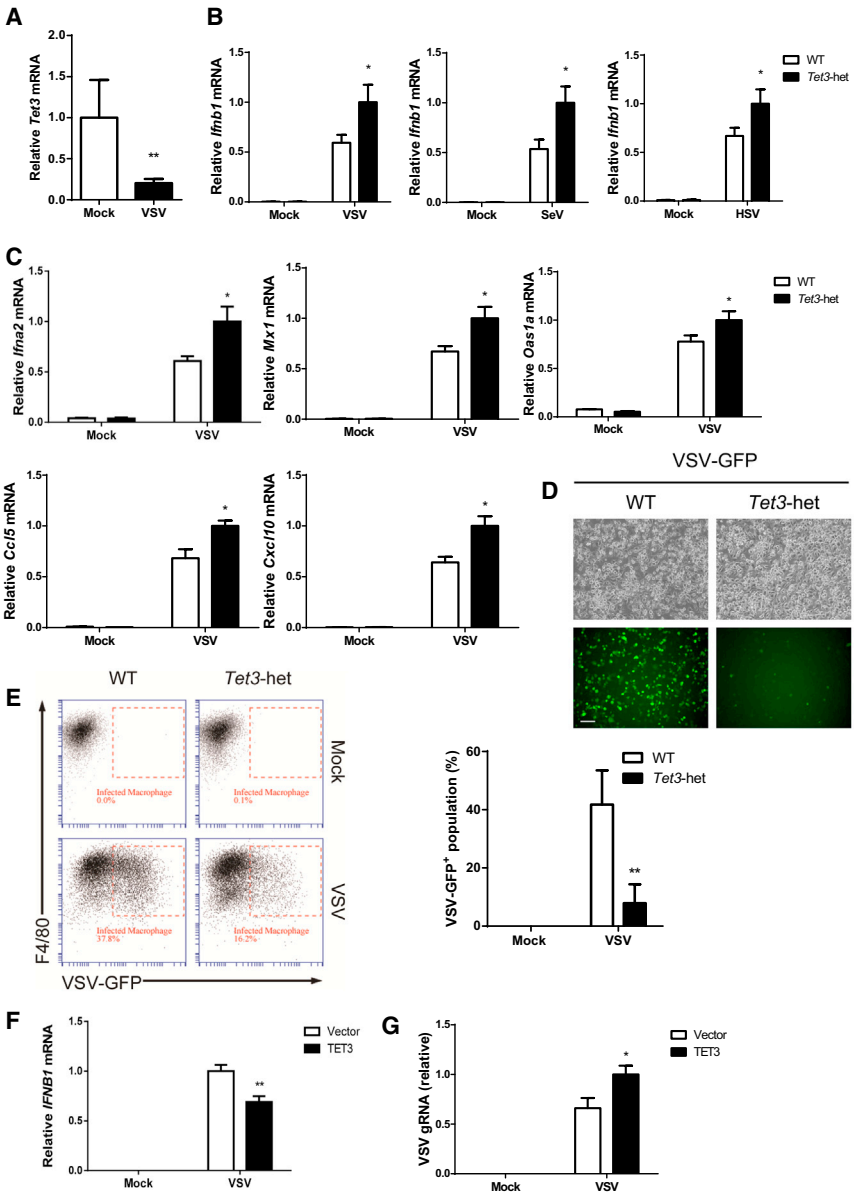

Figure 6. TET3 Inhibits Antiviral Responses

(A) Relative mRNA expression of *Tet3* in PEMs that were uninfected (Mock) or infected with GFP-fused VSV.

(B) Relative mRNA expression of *Ifnb1* in WT and *Tet3*-het PEMs infected with vesicular stomatitis virus (VSV), Sendai virus (SeV), or herpes simplex virus (HSV).

(C) Relative mRNA expression of the indicated interferon-stimulated genes in WT and *Tet3*-het PEMs after VSV infection.

(D) Representative images of the bright field and GFP fluorescence from WT or *Tet3*-het PEMs after VSV infection for 16 hr. Scale bars represent 100 μm.

(E) FACS analysis of the viral load (indicated by VSV-GFP positive cells) in WT or *Tet3*-het PEMs after VSV infection for 16 hr (left). Statistics showing the percentages of VSV-GFP-positive WT or *Tet3*-het PEMs (right).

(F) Relative mRNA expression of *IFNB1* in VSV-infected control or TET3-overexpressing HEK293T cells.

(G) Relative levels of VSV genomic RNA in VSV-infected control or TET3-overexpressing HEK293T cells.

Error bars represent the mean ± SD of at least three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

FACS analysis (Figures 6D and 6E), suggesting better viral clearance in *Tet3*-het macrophages. Consistently, TET3 overexpression in HEK293T cells decreased the mRNA levels of *IFNB1* upon VSV infection (Figure 6F). Furthermore, we observed a higher amount of VSV genomic RNA by qRT-PCR in TET3-overexpressing HEK293T cells (Figure 6G). Taken together, we propose that lower TET3 expression elevates type I IFN production and protects the host against viral infection.

DISCUSSION

Multiple studies have suggested that CpG methylation in gene promoters leads to gene suppression in mammalian cells. Because TET3 mediates DNA demethylation (Hu et al., 2015; Tahiliani et al., 2009), we were surprised to find that TET3 negatively regulates IFN-β production. Previous work has revealed that TET3 can promote microRNA expression, which then accounts for the suppression of target genes (Hu et al., 2014). Although *miR-146a* is a well-known suppressor in the type I IFN pathway, we found comparable levels of *miR-146a* in both WT and *Tet3*-het macrophages. We could not exclude the involvement of other microRNAs, but expression levels of the key signal proteins that are known to regulate type I IFN production were similar in WT and TET3-deficient macrophages. This suggests that TET3 might directly regulate IFN-β production at the transcriptional level. Although the *IFNB* promoter is a non-CpG promoter (Shestakova et al., 2001), we found that TET3 was able to directly regulate IFN-β transcription. Moreover, the oxygenase-dead mutant of TET3 was capable of suppressing IFN-β transcription to levels similar to those found in WT TET3. This further suggests that TET3 might function in a DNA-demethylation-independent manner. Indeed, TET3 interacted and cooperated with HDAC1, a well-known histone deacetylase and gene repressor, which led to the suppression of IFN-β transcription. These findings indicate that TET3 not only regulates gene expression via DNA demethylation but also targets non-CpG promoters by recruiting other binding partners. Our study elucidates the role of TET3 as a scaffolding protein, which bypasses its classical DNA demethylation function and targets non-CpG promoters.

Our findings and those of others together suggest that TET1, TET2, or TET3 could bind to HDAC1 or HDAC2 (Cartron et al., 2013; Williams et al., 2011; Zhang et al., 2015). In addition, the occupancy of HDAC1 in the *Ifnb1* locus was further elevated in the presence of TET3, which was in agreement with the recognized role of HDAC1 in inhibiting IFN-β production (Nusinzon and Horvath, 2006). A recent study has suggested that TET2 interacts with HDAC2 to suppress IL-6 production, which is also independent of DNA demethylation (Zhang et al., 2015). Our study and this finding together suggest that TET3 and TET2 are able to serve as a repressor rather than an activator during gene transcription, which is dependent on HDAC1 or HDAC2, but not on DNA demethylation. It would be interesting to further examine whether this represents a common scenario in the TET family (e.g., whether the TET1-HDAC1 or TET1-HDAC2 complex might also regulate gene expression independent of DNA demethylation). The discovery of the TET-HDAC repressor complex extends our understanding of the coordination of histone deacetylation and methylation for gene expression.

Compared to *Tet1* and *Tet2*, we found that mRNA expression levels of *Tet3* are relatively higher in macrophages. However, the function of TET3 in the immune system was unknown. In this study, we demonstrated that TET3 inhibits the production of type I IFNs in macrophages and dampens the ability to clear viral infections. Upon poly(I:C) stimulation or viral infection, TET3 expression is decreased in macrophages and PBMCs. Considering the negative role of TET3 in type I IFN production, the lower expression of *Tet3* in virally infected macrophages confers an advantage to the host for defending against pathogens. Currently, with US Food and Drug Approval approval of two HDAC inhibitors (HDACis), vorinostat and romidepsin (West and Johnstone, 2014), HDACis are best characterized as anticancer agents because of their ability to reactivate gene expression and inhibit the growth and survival of tumor cells (Johnstone, 2002). Considering that the biological outcome of HDACis depends on the specificity of the HDACis as well as the intrinsic operation of cell-signaling pathways, it would be important to further examine whether the TET3-HDAC1 axis could be a therapeutic target for treating type I IFN-related infectious diseases as well as autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

Tet3-hetrozygous mice were on the 129/B6 background, as described previously (Gu et al., 2011). 8- to 16-week-old littermates were used in this study. All mice were bred under specific pathogen-free conditions at the Animal Care Facility of Shanghai Institute of Biochemistry and Cell Biology (SIBCB), CAS. Animal studies were approved by the institutional animal facility of the Shanghai Institutes for Biological Sciences (protocol IBCB0057) and the National Institute for Viral Disease Control and Prevention.

Macrophage Preparation

BMDMs were derived from bone marrow cells cultured in L929 conditioned medium, and PEMs were harvested from mice injected intraperitoneally (i.p.) with 3.0 ml 3% Brewer thioglycollate medium, as previously described (Zhang et al., 2014). The macrophages were cultured in complete DMEM supplemented with 10% (vol/vol) fetal bovine serum (FBS), penicillin, and streptomycin (100 U/ml).

Antibodies and Reagents

Anti-p-IRF3 (Ser396) and anti-p-TBK1 (Ser172) were from Cell Signaling Technology. Anti-IRF3, anti-Sin3a, anti-β-actin, and anti-GAPDH were from Abcam. Anti-HDAC1 was from Millipore. LPS, anti-FLAG, and anti-HA were from Sigma. Protein G Sepharose was from GE Healthcare. The ELISA kit for IFN-β was from PBL. Poly(I:C) HMW was from InvivoGen. Apicidin was from Cayman Chemical.

Stable Cell Lines

FLAG-tagged murine TET3 (WT, CD, and Mut-CD) was sub-cloned into the retroviral vector pMIGR-IRES-GFP and co-transfected with pCL-10A into HEK293T cells. Retroviral supernatants were collected and used to infect MEFs to generate stable cell lines.

Generation of TET3-Deficient MEFs by Cas9-sgRNA

TET3-deficient MEFs were generated by CRISPR (clustered regularly interspaced short palindromic repeats)-associated protein-9 nuclease (Cas9)-mediated genome engineering as previously described (Zhong et al., 2015). In brief, the pX330-*Tet3*-sgRNA-mCherry plasmid was kindly provided by Dr. J. Li (SIBCB, CAS). MEFs were transfected with the pX330-*Tet3*-sgRNA-mCherry plasmid using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. 48 hr after transfection, mCherry-positive MEFs were sorted with flow cytometry (FACS Aria II, BD Biosciences) and seeded as single cells into 96-well plates. Genomic DNA was extracted from single clones expanded from single cells and was analyzed by Sanger sequencing. One clone with both alleles mutated (Figure S3) was chosen as the representative of the Cas9-*Tet3*-targeted MEFs.

siRNAs and qRT-PCR

SMART pool siRNA probes (Dharmacon) were transfected into PEMs with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Total RNA was extracted from cells with RNAiso Plus (Takara). cDNA was generated with M-MLV transcriptase (Takara) and random hexamers (Sangon). qRT-PCR was performed on a CFX-96 real-time PCR detection system (Bio-Rad) with SYBR Green Master Mix (DBI Bioscience).

ChIP Assay

ChIP assays were performed with the EZChIP kit (Millipore) according to the manufacturer's instructions. In brief, cells were fixed with 0.9% formaldehyde and quenched with glycine. Then, the cells were ultrasonicated with a Bioruptor (Diagenode). The supernatants were collected and diluted, and the indicated antibodies were added for overnight incubation. DNA-protein complexes were immunoprecipitated by protein G Sepharose, followed by washing, eluting, and reverse crosslinking processes. The DNA was then purified and quantified by qRT-PCR.

Dual-Luciferase Reporter Assay

The pGL3-IFN-β luciferase reporter plasmid was kindly provided by Dr. C. Wang (SIBCB, CAS). HEK293T cells were transfected with the IFN-β luciferase reporter, TK-renilla, and the indicated plasmids. The activities of the reporters were measured using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Poly(I:C) Treatment and Viral Infections of Macrophages

Poly(I:C) treatment was described previously (Zhang et al., 2014). In brief, to activate the TLR3 pathway, poly(I:C) was added to the medium to a final concentration of 10 μg/ml. To activate the RIG-I/MDA-5 pathways, poly(I:C) (10 μg/ml) was transfected into cells with Lipofectamine 2000 (Invitrogen). Viral infections in macrophages have been described previously (Du et al., 2015). In brief, unless indicated, virus was added to serum-free medium and infected cells for 1.5 hr. The cells were incubated with virus-free serum-containing medium for another 1.5 hr and then harvested.

Immunoprecipitation

Immunoprecipitation was described previously (Li et al., 2015a). In brief, HEK293T cells or MEFs overexpressing the indicated proteins were washed twice with ice-cold PBS before being harvested in ice-cold lysis buffer

(100 mM KCl, 1% Triton X-100, 2 mM EDTA, and protease and phosphatase inhibitors, pH 7.5). Whole-cell lysates were incubated with the indicated antibodies, followed by protein G Sepharose incubation at 4°C. The Sepharose beads were then washed three times with lysis buffer and resuspended in an appropriate amount of SDS-PAGE loading buffer. The samples were analyzed by immunoblotting analysis.

Statistical Analysis

Statistical significance was determined by a two-tailed Student's t test or a two-way ANOVA with GraphPad Prism 6. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.068.

AUTHOR CONTRIBUTIONS

S.X., C. Liu, X.S., W.L., C.Z., and X.Z. performed experiments; S.X., C. Liu, and X.S. analyzed data. S.X., C. Liu, H.W., C. Li, and X.X. designed experiments. S.X., C. Liu, and H.W. wrote the paper. G.X. provided animals. X.Z., Y.L., J.X., B.S., H.X., and S.L. provided plasmids or designed primers.

ACKNOWLEDGMENTS

We thank Dr. Jisong Li (SIBCB, CAS) for providing the pX330-Tet3 plasmid and Dr. Hui Xiao (IPS, CAS) for providing HSV and VSV. This work was supported by grants from the Ministry of Science and Technology of China (2016YFD05002007 and 2012CB910800), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB19000000), the National Natural Science Foundation of China (31422018, 81571617, 81571552, 31300723, and 81590764), the State Key Laboratory of Cell Biology, SIBCB, CAS (SKLCBKF2013003), and the Instrument Developing Project of the Chinese Academy of Sciences (YZ201339). H.W. is supported by the Hundred Talents Program of the Chinese Academy of Sciences.

Received: February 18, 2016
Revised: May 11, 2016
Accepted: June 15, 2016
Published: July 14, 2016

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